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MÁSTER UNIVERSITARIO EN ZOONOSIS Y UNA SOLA SALUD

Occurrence of Multidrug Resistant Bacteria in aquatic wildlife in Catalonia, Spain.

MASTER´ RESEARCH WORK
Master Zoonoses & One Health
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AKNOLEDGMENT

Bringing up this investigation would've been impossible without the support and teachings from my director, Laila Darwich and the daily help and guidance of Chiara Seminati that made the laboratory work for my TFM the best part of my entire day, to both I will be forever thankful. To all the teachers involved on my education during this period for their effort on teaching and their compromise with the students even on the current exceptional conditions. Finally, but the most important, I want to thank my mother, Teresa, for making everyone of my dreams possible and supporting me on my never ending new professional goals, to her, for her, for us.

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ABSTRACT

Multidrug resistance on bacteria is one of the principal public health threats of the 21st century with severe social implications and high economical burden all over the world. Even if the resistance generation is per-se a natural evolutionary process, the regular and repeated release of AMR bacteria and AMR determinants into natural ecosystems imposes extra selective pressure that has leaded to an unprecedented emergence of MDR, XDR and PDR bacterial strains. Aquatic ecosystems have shown to be a major transmission media and all kinds of antimicrobials have been detected in different aquatic environment samples. On our study 96 aquatic-related wild animals (33 reptiles and 63 mammals) from the Wildlife Rehabilitation Center of Torreferrussa were sampled. Rectal or cloacal swabs samples were taken and cultured in antibiotic selective media for microbiological identification and antibiosensitivity testing. A total of 36 bacterial spp from invasive turtles and 22 from aquatic-related mammals were isolated. The recovered species (N=58) have clinical significance on emerging opportunistic nosocomial and foodborne infections: *E. coli* (N=13), *Aeromonas* spp. (N=10), *Pseudomonas* spp (N=9), *Burkholderia cepacia (N=4), Citrobacter, Moraxella* spp., *Ralstonia picketti* (N=3); *Salmonella, Serratia, Klebsiella and Proteus* spp. (N=2) and single *Bordetella bronchiseptica, Mannheimia haemolitica, Morganella morganii, Providencia rettgeri, Rahnella aquatilis and Rhizobium radiobacter.* 100% hold MDR profiles, 24% fit on the XDR category, moreover, *Burkholderia cepacia* (N=2) and *Moraxella* spp. (N=1) where PDR bacteria. All things considered, aquatic wildlife might not serve only as reservoirs and vectors for MDR, XDR and PDR, but also as highly effective surveillance targets to determine the extent of water-sources and neighbouring areas AMR pollution and the early detection of novel MDR profiles on bacteria of medical relevance.

INTRODUCTION

Antimicrobial resistance emergence

The development of antimicrobial agents during the 20th century made far-reaching changes on the management and therapy of bacterial infections. After 1940s when the fist antibiotics (Penicillin and Streptomycin) were introduced, infections what that used to be fatal became curable and ever since, antibiotics have saved millions of lives (1,2). With in these 80 years, the importance of antibiotics has become even greater, they are used to treat a wide range of bacterial infections in humans, animals and plants and they are an indispensable part for numerous lifesaving medical procedures such as: organ transplants and chemotherapy protocols, the care of preterm babies and all sort of surgical procedures success depend on them. However the emergence of antimicrobial resistance (AMR) is now jeopardizing all those achievements by compromising antibiotic´s effectiveness and questioning their future utility (3).

Neither antibiotic production nor antibiotic resistance (ABR) are new phenomena. AMR has existed for millions of years as an unavoidable evolutionary consequence of microbial competition in the environment that occurs as a selection process by which bacteria acquire some degree of resistance to face natural challenges (4,5). Premised upon this fact, natural habitats have harboured antibiotic resistance genes independently of human activity, nevertheless, AMR have emerged as one of the principal public health threats of the 21ST century, and especially urgent regarding ABR in bacteria which is largely attributed to their over and/or inappropriate usage (6) . While "evolution" is a wider term that explains a gradual change, "emergence" more specifically, incorporates key components of the evolutionary process: mutation, recombination and horizontal gene transfer (HGT) by which bacteria may acquire resistance to antibiotics (7) resulting on the current unprecedented and mounting variety of resistant organisms, their global geographic distribution, high-speeded spread and the broad pool of the resistance in one simple organism (multidrug resistant microorganisms MDR) (8).

Impact and One Health relevance

All over the world antibiotic resistance is rising at dangerous rates, making common infections become harder and sometimes impossible to treat, therefore, life threatening (pneumonia, tuberculosis, gonorrhoea, foodborne diseases or a simple cystitis) (9). In addition, economic burden and social repercussions must be taken into consideration.

Increased resistance leads to extended hospital days, isolation wards, stringent infection control measures and treatment failures; on the social costs death and loss of productivity are included (6). The European Commission claimed that costs attributed to resistant bacterial infections amounted to €1.5 billion annually (10) and The United States healthcare systems had estimated that the additional cost of antimicrobial-resistant infections reaches 20 billion dollars plus productivity losses of US$35 billion per year (11). Furthermore, it´s been estimated that drug- resistant infections might cause 10 million human deaths annually by 2050, with total costs by this date of US$100 trillion in lost output if not addressed with urgency (12).

Resistant microorganisms exist in humans, animals, food and the environment, representing a global problem of a complex epidemiology. The entangled roles played by humans, pet animals, livestock and wildlife along with their shared ecosystems; needs to be faced on a broad, integrated approach. The One Health approach considers all underlying factors and the institutional context at all levels of society, it is therefore not just suited, but essential to embrace AMR research and surveillance efforts under this perspective (13,14).

Role of aquatic ecosystems

The emergence and spread of antimicrobial-resistant bacteria (AMRB) in natural environments is a matter great concern with serious implications; the accelerated use of all known antimicrobials for the benefit of human, animals and agriculture conclude their regular and repeated release into the environment and natural ecosystems (15). Even though, it is difficult to disentangle the transmission routs of AMRB there is increasing research pointing that water might be a major transmission media; all kinds of antimicrobials have been detected globally in different aquatic environment samples. Besides, there is abundant evidence that the exchanges of resistance genes between environmental bacteria and the human pathogens occurs in these aquatic systems (2,16).

Run-off from farms, slurry tanks and manure-fertilised fields as well as hospital, pharmaceutical and veterinarian sewage effluents result in the contamination of surrounding water sources and land with antimicrobial drugs and antimicrobial-resistant bacteria (4,17). Antibiotics and its metabolites have been reported in, wastewater treatment plants (WWTPs) effluents, WWTPs bio solids, soil, surface water, ground waters, sediments, biota and even drinking water. Sewage effluents provide the rout for the dissemination of bioactive

compounds and resistance gens into natural bacteria ecosystems making WWTPs a hotspot for HGT between bacteria of different origins, imposing a selective pressure upon target and non-target bacterial population disturbing key bacterial cycles and mechanisms critical to maintain aquatic balance (15,18).

Not even the most modern sewage treatment plants remove all antibiotic residues nor the AMR bacteria, therefore they end of into rivers downstream. Correspondingly, the effects of this pollution, affects wild fauna, which would not ordinarily be exposed to these antimicrobial agents, acquiring AMR bacteria by inhabiting, feeding and/or drinking from AMR polluted environments (3,16) increasing the risk of MDR bacteria carriage in water-associated species that may serve as reservoirs and melting pot of bacterial resistance.

OBJETIVES AND IMPORTANCE

Once AMR bacteria colonize aquatic wild animals, in turn they end up playing a triple role; they become new environmental reservoirs, vectors providing a biological spread mechanism and bio-indicators (sentinels) of resistant bacterial pathogens and genetic determinants of AMR in the environment. The fist two roles, oppose an evident hazard for human and animal health by closing the cycle with the transmission of resistant strains to waterways, raw food products in fields and human-associated environments via faecal contamination or direct contact with pets and humans (e.g. trapping or hunting, wildlife veterinarians) etc. (1,16).

Given that 60% of emerging infectious diseases are zoonosis, of which 70% originated in wildlife and that aquatic ecosystems have show to provide an ideal matrix for the acquisition and dissemination of AMR determinants, antibacterial resistance in aquatic wildlife represents a potential public health threat (8) that is why, elucidating the origins of antimicrobial resistance in the wildlife is by itself important because of the its zoonotic implications. Yet, it is upmost important their potential as sentinels which will provide information about the patterns of distribution, risk of exposure and level of environmental contamination by AMR bacteria and motile genetic elements (MGEs) encoding antimicrobial resistance, therefore enhancing AMR surveillance efforts.

On this study, samples of native and non-native aquatic-related wildlife arriving to the Catalonian Torreferrusa Wildlife Rehabilitation Centre were taken in order to determine the occurrence of antibiotic and multidrug resistant bacterial strains harboured by these species which might help to determine their roles as resistant bacteria or resistance determinants reservoirs, suggest the implications of antimicrobial pollution on aquatic ecosystems and/or consider their importance as sentinels of AMRs.

METHODOLOGY

Sampling procedure

Subjects sampled corresponded to wild species belonging to aquatic-related ecosystems arriving to the Wildlife Rehabilitation Centre of Torreferrusa (WRC) (Catalonia – Spain), between April 2019 and May 2020. This is a public WRC under the direction of the Catalan Wildlife-Service (Direccio´ General de Polı´tiques Ambientals, Departament de Territori i Sostenibilitat of the Generalitat de Catalunya). Sampling methods and handling protocols of animals were in agreement with the Catalan Wildlife Service who stipulates the management protocols and Ethical Principles according to the Spanish legislation. Samples, rectal or cloacal swabs, were collected upon animal´s arrival before they were summited to any medical treatment or exposed to contact with other animals at the facility. The sampling of some aquatic mammals (mainly *Neovison vison*) was conducted from frozen non-treated carcasses. All samples were taken using sterile swabs, placed on transport media and then taken to the veterinary faculty of the Autonomous University of Barcelona, where they were processed.

Bacterial characterization & antimicrobial susceptibility testing

Samples were then plated on two MacConkey agar media enriched with ceftriaxone (1mg/L) and colistin (2mg/L), and also on XLT4 selective media for *Salmonella* spp., each. Single colonies growing on the plate were then subcultured on regular MacConkey agar and then identified biochemically using API (NE20/E20) system (bioMe´rieux, Marcy l'Etoile, France).

Once identified, bacteria were plated on Muller-Hilton agar for antimicrobial susceptibility testing using the Kirby-Bauer/disk-diffusion method according to the Performance Standards

for Antimicrobial Susceptibility Testing for bacteria isolated from animals (19) and from humans (20) for drugs not licensed for veterinary use.

Each bacteria was tested for 13 different antimicrobials that represent a wide range of antibiotic classes of medical and veterinary interest: Amoxicillin/ Clavulanic acid (AMC), Ampicillin (AMP), Cefquinome (CEQ), Ceftriaxone (CTX), Enrofloxacin (ENR), Ciprofloxacin (CIP), Gentamicin (GEN), Lincomycin/ Spectinomycin (LS), Erythromycin (ERY), Tetracycline, Chloramphenicol (CPL), Colistin (CST) & Trimethoprim/sulfamethoxazole (SXT)(Annex 1).

Based on the lab testing readings, isolates were classified as Susceptible, Intermediate or Resistant. For statistical assessments, all isolates that exhibited intermediate resistance were re-classified as resistant. In addition, multidrug resistance (MDR) was defined as resistance to at least one agent in ≥3 antimicrobial categories; extensive drug resistance (XDR) as resistance to all but two of the tested antimicrobial categories and finally pan-drug (PDR) as resistance to all the categories tested (21) .

Additionally, identified bacterial DNA was extracted and frozen for conservation for further resistance genes analysing. Refer to (Annex 2) for process diagram.

RESULTS

Selection and identification

Out of a total of 96 animals (33 reptiles and 63 mammals) sampled at Torreferrusa Wildlife Rehabilitation Centre. Within the given period, 58 bacterial colonies were obtained after the selective media initial screening, predominantly from invasive reptile species 36(62%) and aquatic mammals 22(38%) (Figure 1a.), including:

Reptiles:

- Pond slider turtle *(Trachemys scripta*) 35(60%), an invasive species that inhabits water areas like ponds, lakes, swamps, creeks, streams, or slow-flowing rivers with abundant aquatic plants that they feed from (22).

- Peninsula cooter (*Pseudemys peninsularis*) 1(2%), also considered invasive species, is a freshwater turtle that inhabits mainly slow moving or stagnant waterways with abundant basking sites, submerged vegetation, and sandy bottoms (23).

Mammals:

- American Mink (*Neovison vison)* 17(29%), a member of the Mustelidae family, is a semiaquatic native to North America that is considered as invasive species in European freshwater ecosystems (24) .
- Eurasian otter (*Lutra lutra*) 5(9%), also from the Mustelidae family, found in the waterways and coasts, forage in water and nest on land. They inhabit rivers, lakes, streams, freshwater and peat swamp forests, rice fields, ocean shores, fjords, caves, and terrestrial habitats adjacent to waterways (23) (Figure 1b).

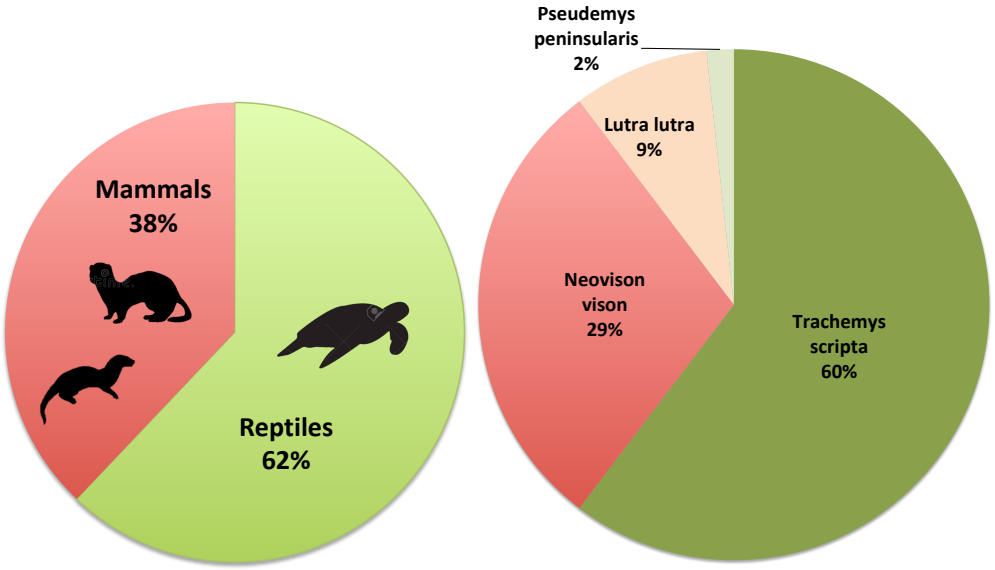


Figure 1. Samples distribution according to animal group (a) and detailed distribution for each animal species.

Among 58 bacterial colonies recovered, 22 different bacterial species were documented; Enterobateriales represented the 46%(27), being *E. coli* predominant among them. The 54% (31) left, corresponded to different scatter species from Pseudomonadaceae 12(21%), Aeromonadaceae 10(17%), Burkholderiales 7(12%), Pasteurellaceae 1(2%), and Agrobacterium 1(2%), families (Figure 2).

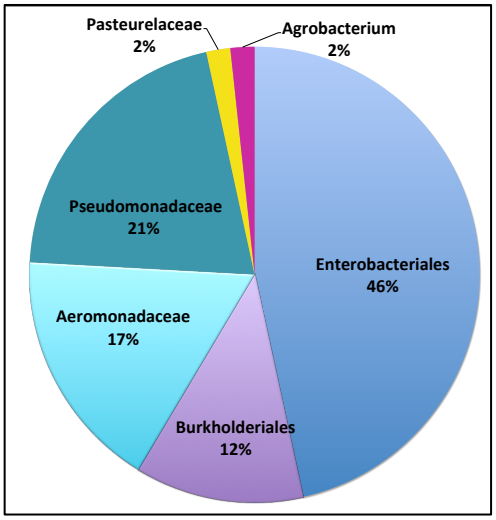


Figure 2. General distribution of bacterial families

Apart form the fact that all but one (the peninsula cooter) animals hosted more than one AMR bacteria species, pond slider turtles showed the broader bacterial diversity (15/22) compared with the mammals; the American mink was the source of 10 out the 22 species found while the Eurasian otter only 3. The bacterial distribution showed *E. coli* strains to be evenly distributed on mammals and reptiles, other enteric bacteria like *Salmonella* spp, *Morganella morganii* and *Providencia rettgeri* was only found in mammals while, *Aeromonas* spp. and *Burkholderia cepacia* were exclusive of turtles (Table 1).

Family	Genus	Species	TS %	PP %	NV %	LL %
Enterobacteriaceae (Enterobateriales) 21	*Escherichia*	*E. coli*	46	7	46	-
	Salmonella	*Salmonella enterica* subsp. *arizonae*	-	-	100	-
		Salmonella spp.	-	-	100	-
	Klebsiella	*Klebsiella* spp.	100	-	-	-
	Citrobacter	*C. freundii*	100	-	-	-
		C. diversus	100	-	-	-
	Rahnella	*R. aquatilis*	100	-	-	-
Yersiniaceae (Enterobateriales) 2	*Serratia*	*S. fonticola*	100	-	-	-
		S. marcescens	100	-	-	-
Morganellaceae (Enterobateriales) 4	*Proteus*	*P. vulgaris*	50	-	50	-
	Providencia	*Providencia rettgeri*	-	-	100	-
	Morganella	*M. morganii*	-	-	100	-
Burkholderiaceae (Burkholderiales) 6	*Burkholderia*	*B. cepacia* complex	100	-	-	-
	Ralstonia	*Ralstonia picketti*	50	-	50	-
Alcaligenaceae (Burkholderiales) 1	*Bordetella*	*B. brochiseptica*	-	-	100	-
Pseudomonadaceae 12	*Pseudomonas*	*P. fluorescens*	-	-	50	50
		P. putida	100	-	-	-
	Moraxella	*Moraxella* spp.	33	-	33	33
Aeromonadaceae 10	*Aeromonas*	*A. hydrophila*	100	-	-	-
		A. hydrophila/caviae	100	-	-	-
Pasteurellaceae 1	*Mannheimia*	*M. haemolytica*	-	-	-	100
Rhizobiaceae 1	*Rhizobium/ Agrobacterium*	*R. radiobacter*	100	-	-	-
TS- *Trachemys scripta*/ NV - *Neovison vison* / LL - *Lutra lutra* / PP - *Pseudemys peninsularis*						

Table 1. Bacterial families and reported corresponding species recovered from the animals sampled.

Antimicrobial susceptibility findings

All the bacteria reported on our study fulfil the criteria to be considered MDR (Figure 3a). MDR-Enterobacteria represented the 59% of all the species of this family that were isolated (16/27). Among all the rest families, 48% were MDRB leaded by the *Aeromonas* and *Pseudomonas* species. 41% of the total 58 collected species fit also as XDR-bacteria, but mostly belonged to non-enterobacteria strains (13/24, 54%), namely *Burkholderia cepacia* and *Pseudomonas* spp, but it important to remark that more than half (6/13) of the *E. coli* and

all *Salmonella* strains collected on this study were XDRB. A total of 3 PDR-bacteria were reported, also corresponding to the same two species mentioned before (Figures 3b, 4). Generally speaking, MDR, XDR and PDR-bacteria were equally found on reptiles and mammals, in fact, two out of the three PDRB collected corresponded to *Trachemys scripta* and one to *Lutra lutra* inviduals (Figure 3c).

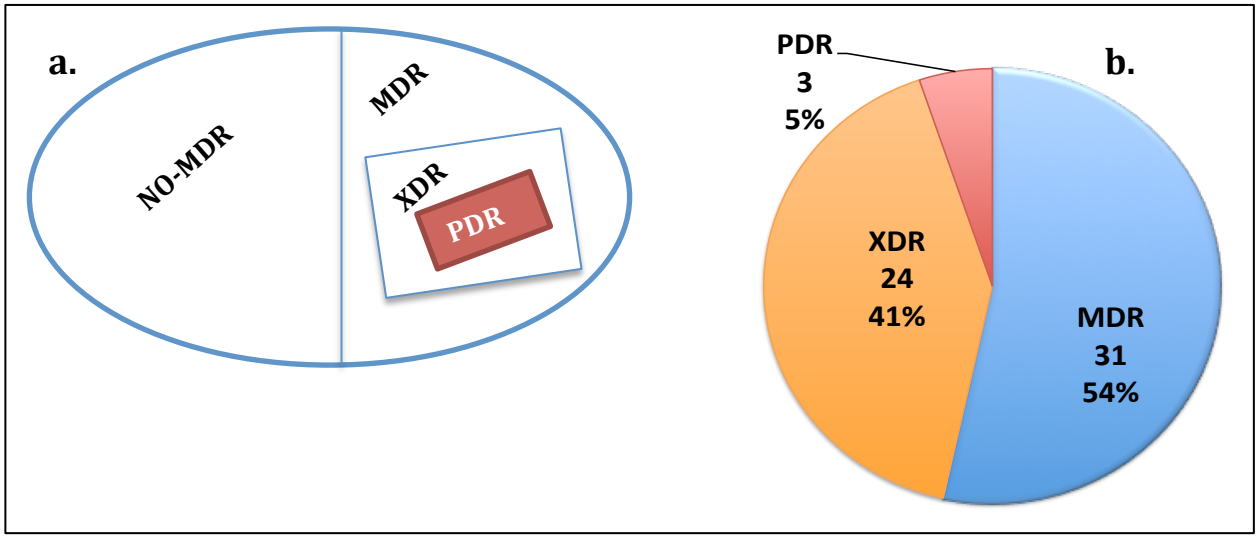


Figure 3. Diagram of MDR, XDR and PDR relationship (a) (21) and their overall occurrence (b).

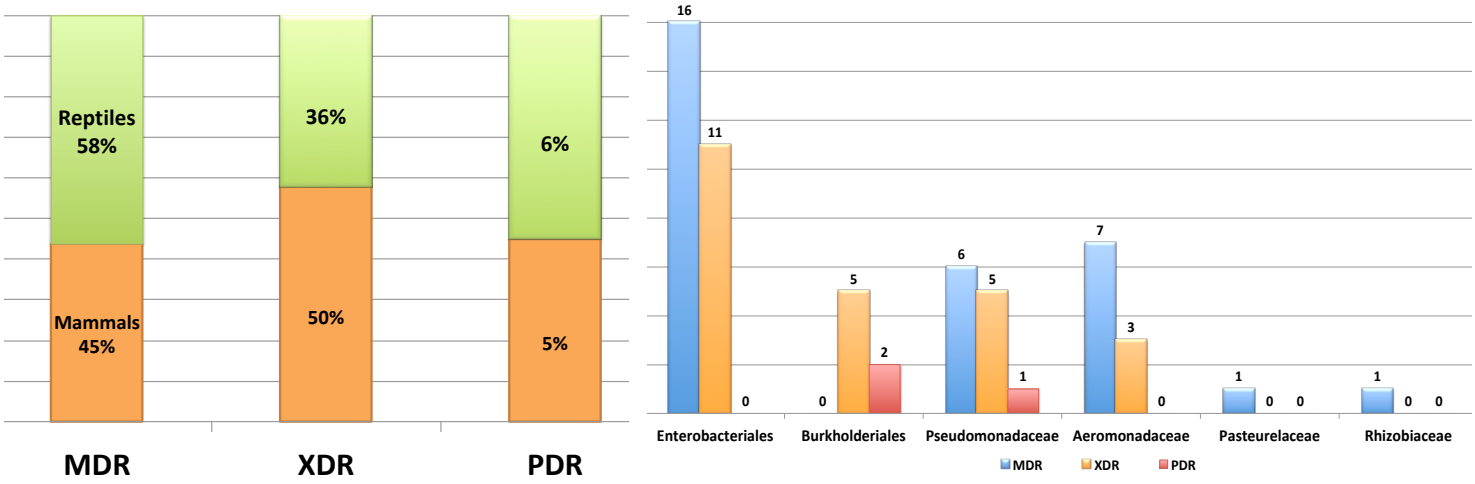
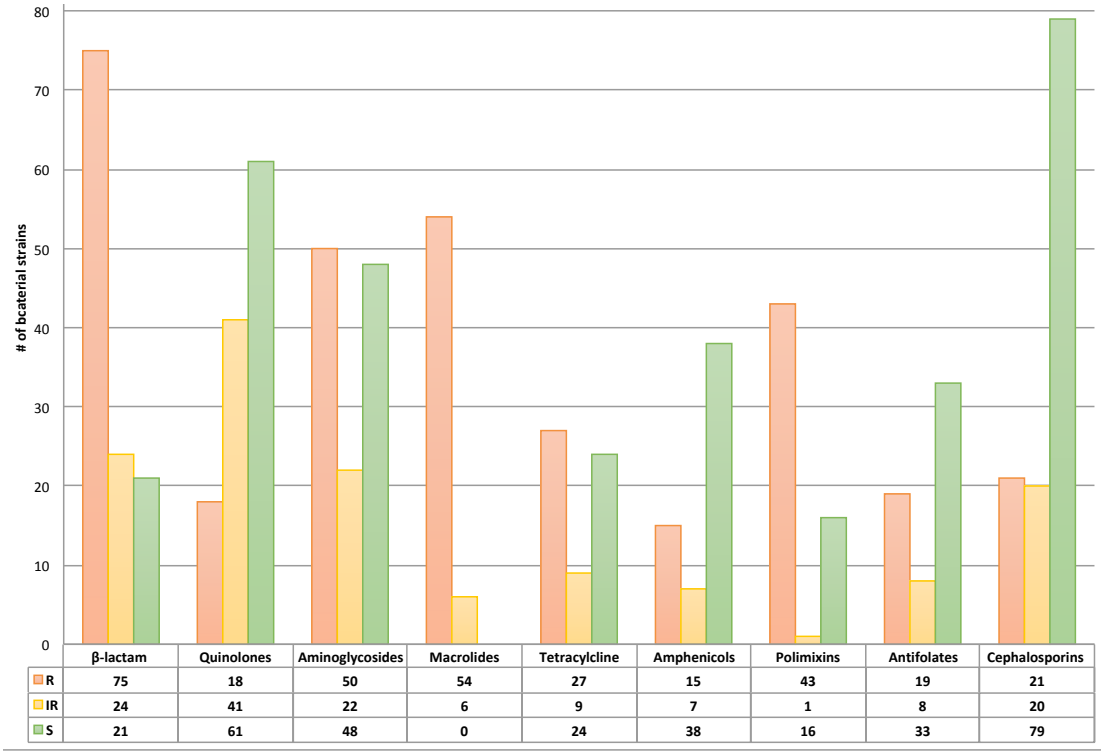


Figure 4 MDR, XDR and PDR distribution per animal (left) and bacterial group (right).

In total we obtained 754 analysed results, 448 corresponded to complete or intermediate resistance (I+R), and only 306 susceptibility. The highest resistance was hold by the β-lactam group (13%) not closely followed by aminoglycosides and macrolides, 10% and 8%, respectively (Figure 5). The specific antibacterial with highest resistance overall was Erythromycin (58/58) followed by Ampicillin (51/58) and Colistin (44/58); while 3th generation cephalosporins and fluoroquinolones exhibited the lowest on both, mammals and

reptiles. Colistin-resistant strains were more abundant on mammals than reptiles, while Ampicillin resistance was higher on reptiles (Figure 6).



	β-lactam	Quinolones	Aminoglycosides	Macrolides	Tetracylcline	Amphenicols	Polimixins	Antifolates	Cephalosporins
R	75	18	50	54	27	15	43	19	21
IR	24	41	22	6	9	7	1	8	20
S	21	61	48	0	24	38	16	33	79

Figure 5. Resistance distribution among all the antimicrobial families tested.

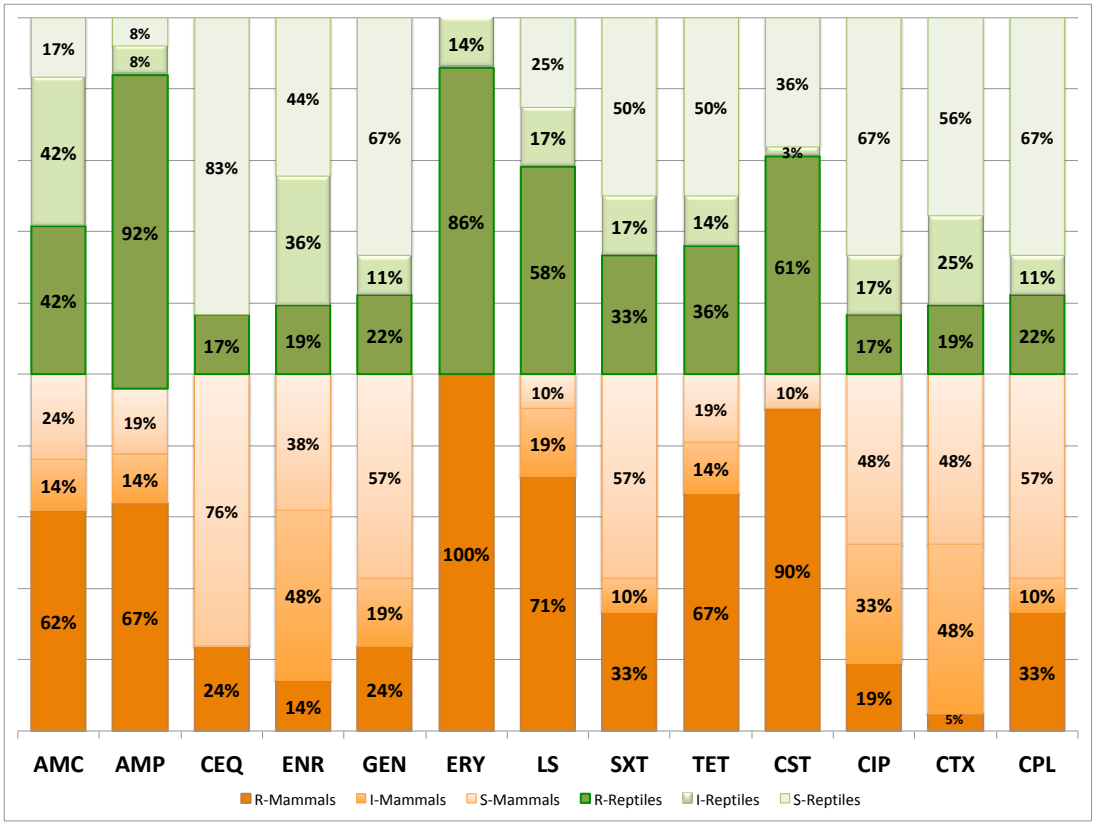


Figure 6. Levels of susceptibility (IR, R, S) recorded per antimicrobial drug. Green representing reptiles and orange mammals. Amoxicillin/ Clavulanic acid (AMC), Ampicillin (AMP), Cefquinome (CEQ), Ceftriaxone (CTX), Enrofloxacin (ENR), Ciprofloxacin (CIP), Gentamicin (GEN), Lincomycin/ Spectinomycin (LS), Erythromycin (ERY), Tetracycline, Chloramphenicol (CPL), Colistin (CST) & Trimethoprim/sulfamethoxazole (SXT).

Finally, considering each bacterial AMR profile, they resulted very different between families, however, profiles corresponding to different species among the same family resulted very concordant with one and other as seen with Burkholderiales (Figures 7,8). Contrastingly, the Enterobateriales show high diversity of AMR profiles between species, being the *Serratia* spp. the one holding the most different profile but also holding the lowest spectrum AMR pool of all.

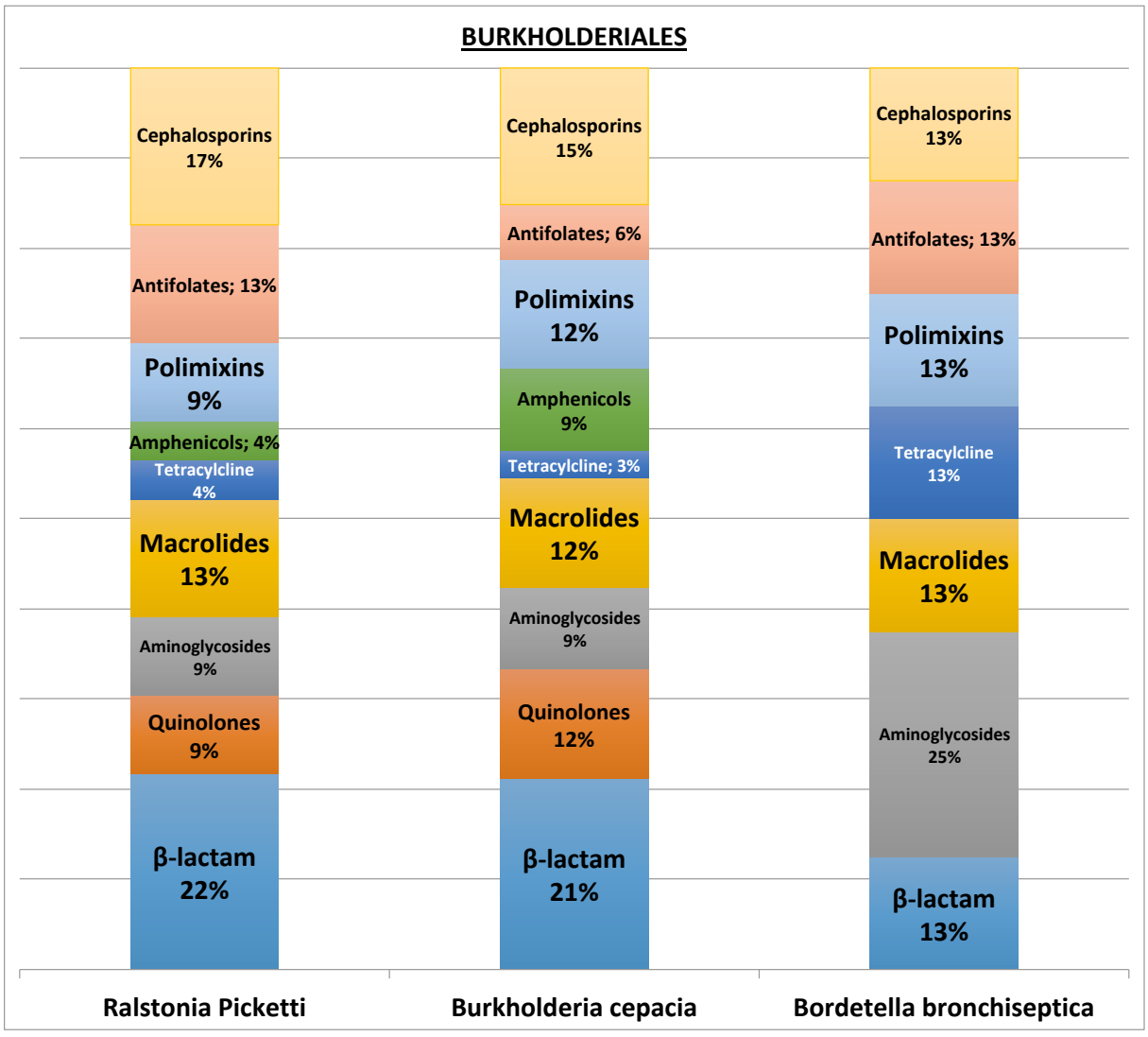


Figure 7. Antimicrobial Resistance Profiles between the Burkholderiales members from left to right, *Ralstonia picketti, Burkholderia cepacia and Bordetella bronchiseptica.*

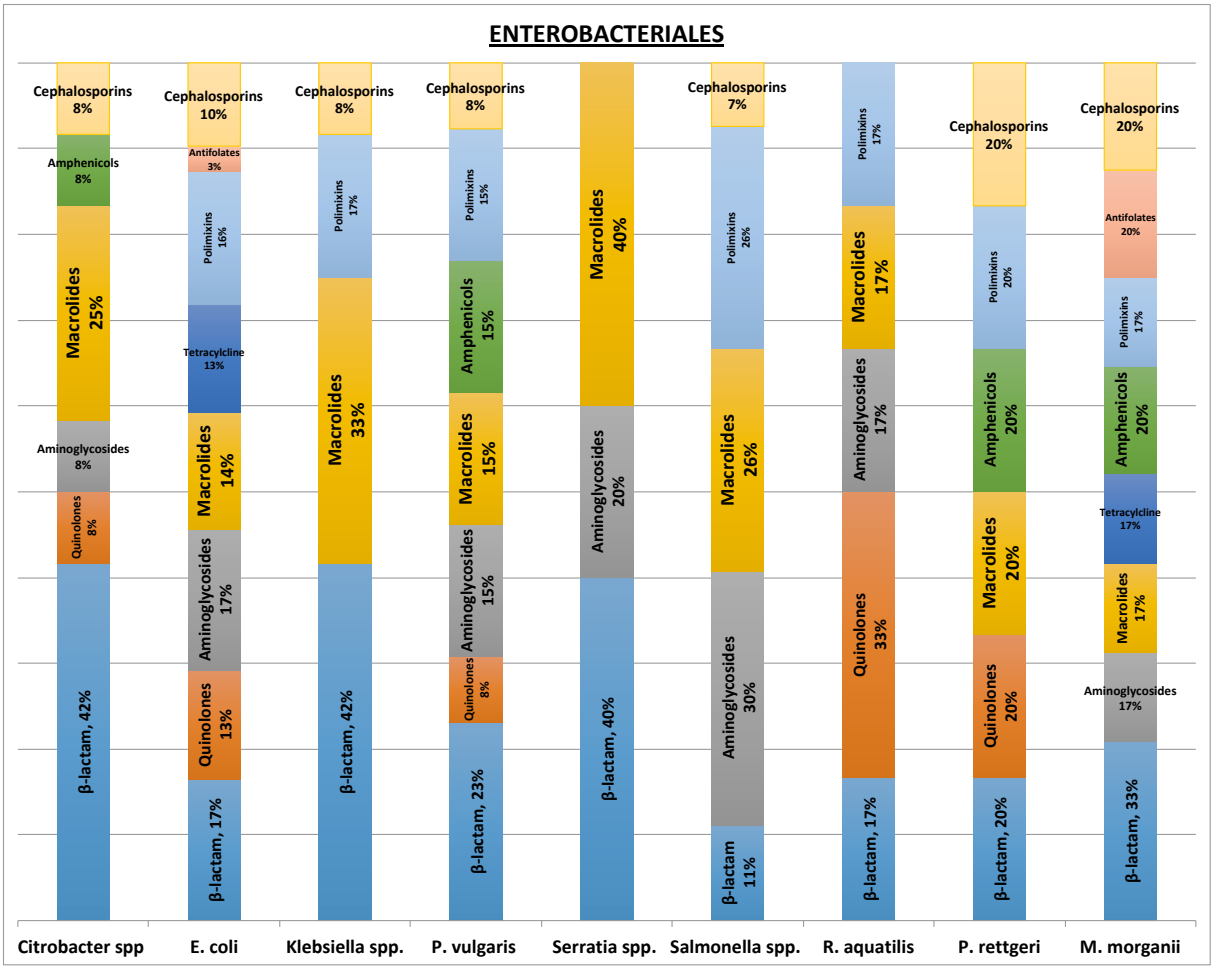


Figure 8. Antimicrobial Resistance Profiles of each enterobacteria species.

Other considerations

Intrinsic resistance was also considered; only the 12% (55) of the overall resistance could be attributed to bacterial intrinsic characteristics (Annex 1). Additionally, two EUCAST AMR interpretation rules with relevance to this study were considered, rules 12.8 and 13.8. Even though, such implications weren't taken in consideration into our overall results, given its low matter-of-course significance, when the interpretations have a therapeutic aim, they must be considered.

12.8 - If IR to gentamicin but R to other aminoglycoside, then report as resistant to gentamicin.

13.8 – If resistant to ciprofloxacin, then report as resistant to all fluoroquinolones.

RESULTS DISCUSSION

Enterobacteria are ubiquitous of many environmental habitats; besides, they are commensal microbiota of mammals, birds, some reptiles and also invertebrates. Nevertheless, some of

them have emerged as important causes of nosocomial and community-acquired antibiotic resistant pathogens harbouring important transmissible AMR characteristics (16). Our results showed an extensive variety of primordially, Enterobacteriaceae and other commensal microbiota species on the fauna sampled, with a predominance of resistant *E. coli* strains. Furthermore, the all bacteria isolates recovered and tested showed a concerning broad antibiotic resistance spectrum.

On a general basis, *Escherichia coli* is part of the normal intestinal flora in healthy humans and animals, and most of its strains are either harmless or produce very mild, self-limiting diarrhoeal episodes. But, it is also among the most frequent causes of urinary tract (UTI) and bloodstream infections worldwide. Not to mention, *E. coli* infection treatment has been increasingly complicating due to the emergence of resistance to most of the first-line antimicrobial agents (25).

The major concern is the resistance to third generation cephalosporins and carbapenems mainly conferred by enzymes known as extended β-lactamases (ESBLs) and carbapenemases; features that have also severely compromised the effectiveness of Ampicillin and Tetracycline (25,26). On this regard, the *E. coli* colonies on this study still hold an important susceptibility, 1 and 6 out of the total 13, towards CEQ and CTX, respectively. On the other hand, AMP and TET showed very low susceptibility, with 9 & 11 out of the 13 testing resistant to these last antimicrobials. Nevertheless, the highest occurrence of resistance was recorded for Colistin, 13 out of 13, a worrying finding since other studies find CST to present the lowest resistance prevalence (27) and considering its "last-resort" status on human medicine and its critical role on veterinary gastrointestinal infections, specially on pigs and cattle (28). Finally, our findings do posit CEQ as the most effective against our *E. coli* strains followed by SXR (5/13).

The second most abundant bacteria recovered corresponded to genus *Aeromonas.* Motile *Aeromonas* spp. are pathogens of clinical importance of obligatory notification to PAHO (Pan-American Health Organization) in case of outbreak. The most important are: *A. hydrophila* the most virulent, *A. hydrophila/caviae*; the two species found on this study, and *A. veronii* biovar *sobria* which was not present. *A. hydrophila* is found in all freshwater environments including chlorinated water. The bacterium is transmitted to humans through oral contact with contaminated foods, water, soil, faeces and ingestion of contaminated fish or

reptiles causing severe gastroenteritis or can cause bacteraemia and soft tissue infections even meningitis when acquired through open wounds exposed to contaminated water or handling contaminated fauna. *A. hydrophila* is mostly related to the ingestion of contaminated fish, vegetables or their sub products, while *A. caviae* has only been related to vegetables (29–31)

PAHO treatment guidelines indicate that *Aeromonas* spp. are sensible to SXT, CPL and GEN and resistant to AMP. These recommendations mostly match with our screening; although some resistance were found towards SXT and GEN, none presented resistance to CPL. Accordingly, all *Aeromonas* spp. on this study (10/10) were resistant to AMP. Other studies have shown the failure of treatment of wound infections when ERY was prescribed which resolved after switching to CIP (32), our findings also show lower resistance to CIP compared with ERY, 6/10 R and 9/10 S, respectively.

Third most abundant was the *Pseudomonas* genus, *Pseudomonas fluorescens,* 7 and *Pseudomonas putida,* 2; both water and soil ubiquitous bacteria that used not to be considered as pathogens. However over the past three decades they have emerged as uncommon opportunist pathogens capable of nosocomial infections with MDR characteristics and high mortality rates. It has also been implicated in fish disease "Fin Rot" (*Pseudomonas fluorescens)* often observed in aquaria and aquaculture; bird cases of necrotizing hepatitis and wound infection in horses have also been reported (*Pseudomonas putida)* (33–37). Even if it is a growing concern regarding the resistance shown to first line β-lactam, some 3th generation cephalosporins and chloramphenicol; our study reported complete susceptibility to CTX, on the other hand a high resistance was exhibit to CPL outstripped just by ERY, all resistant. Treatment protocols including CIP, GEN & CST have been successful, (38,39) as a matter if fact, all those showed very low to none resistance at all on our screening.

Even if not greater in abundance but have crucial implications, were the two Salmonella spp. recovered. *Salmonella* are one of the most studied AMR related bacteria on wildlife, only before *E. coli* and *Campylobacter*; given its zoonotic label, it is of public health significance, with considerable economic impact and one of the most common food-borne pathogens. The pathogen is widely host-adapted and ubiquitously present at all levels of human food chain which status has been aggravated since antibiotic-resistant clones are frequently implied as the etiological agents in these outbreaks leading to treatment failures, higher risk of bloodstream infections, and increased rate of hospitalizations. Many reports point different

MDR *Salmonella* strains, in our case, all exemplars were XDR, however very low resistance was recorded towards 3th generation cephalosporins, fluoroquinolones and chloramphenicol, which is comforting. (40,41). It is also pertinent to remark that among the 3 *Salmonella* strains, one was fully identified as *Salmonella enterica* subsp *arizonae* a common gut inhabitant of reptiles that has sporadically been associated with mild to severe infant gastroenteritis, otitis, pulmonary and joint sepsis some of which have directly been traced to turtle or snake handling (42–44).

Less present bacteria included: *Klebsiella* spp (2); *Burkholderia cepacia* (4); *Moraxella* spp and *Ralstonia picketti* (2), *Citrobacter freundii* (2) and *diversus* (1), *Proteus vulgaris* (2) and single strains of *Serratia marcescens* and *fonticola*, *Bordetella brochiseptica, Mannheimia haemolitica, Providencia rettgeri, Rahnella aquatilis, Morganella morganii and Rhizobium radiobacter*. All of them with evidence of threat to human and animal health, being implicated on mainly nosocomial opportunistic infections ranging from wound to urinary track, respiratory tract and bloodstream infections to severe septic shocks and meningitis. Besides all showed broad MDR pools that have also been reported by other studies, the following shows detailed information regarding each particular health implications and AMR pools compared to previously documented.

Table 2. Summarized clinical relevance, MDR classification and comparing previous AMR findings on the lesser-recovered bacteria.

Bacteria	MDR	Clinical relevance	Source	Previously documented antibiotic susceptibility **	Susceptibly findings
***Klebsiella* spp.**	1/2	Healthcare-associated Pneumonia Sepsis Wound infections Urinary tract infections Meningitis	(45–48)	AMC 75%R GENT 45.8%R CIP 87.5%R SXT 95.8%R TET 58.3%R AMP* CST, S	All IR All S All S All S All S AMP and CST R 100% R to ERY
Burkholderia cepacia	4/4 2XDR 2PDR	*B. cepacia* complex affect mostly cystic fibrosis patients, nosocomial but also environment related infections. Major players of MDR. The "cepacia syndrome": necrotizing pneumonia, sepsis and overall negative prognosis.	(49)	Treatment relays on extended-spectrum cephalosporins CLP* 50%R SXT *50% R CIP 50% R AMC* 50% R TET 50% R	CEQ 50% R CTX 75%R 75% R 50% R 50% R 75% R 225% R AMP*, CST &ERY 100%R

Citrobacter freundii & diversus	3/3 1XDR	Commonly found in water increasingly becoming MDR Encephalitis in sheep with high mortality. Common human UTI infection Diarrhoea Sepsis	(50–53)	CIP 32% Aminoglycosides CPL 23% TET 41% AMC* AMP*	100% S GEN 100% S LS 66,6% S 66,6% S 66,6% S 100% R 100%R
Ralstonia picketti	2/2 2XDR	Emerging nosocomial bloodstream infections on immunocompromised patients due to contaminated medical solutions.	(54–56)	Aminoglyc. - R β-lactam – R Polimixins - R SXT – R CIP – R	GEN-S, LS-R AMC & AMP – R CST 50% R 100%R 50%IR Cephalosporin R
***Moraxella* spp.**	3/3 2XDR 1PDR	Commensal of URT, but cause of chronic bronchitis and pneumonia went colonizes the LRT of immunodeficient patients. Some strains are with ocular infections in cattle.	(57,58)	Considered susceptible to AMP, AMC, GEN, and 3th Cephalosporin. Macrolide 75%R CIP 75%R	100% R to AMP, AMC, GEN. CTX 100% IR CEQ 66.6% R ERY 100% R IR and S – CIP SXT, LS 100% R
Serratia marscences & fonticola	1/2	The less common on human faecal flora. At early 20th century still non-pathogenic, now full spectrum human clinical disease harbouring MDR. UTI, Surgical incision infection neonatal sepsis & meningitis	(59,60)	β-lactam R Susceptible to CTX, GENT & CPL CST* AMC*	AMP 100%R * All S to CTX and also CEQ, CPL. *S. marcescens* IR to GEN and LS. Both R to ERY 100%S 100% S
Proteus vulgaris	2/2 1XDR	Nosocomial UTI infections related with long-term care facilities. Cystitis Bacteraemia	(61)	CIP, S TET* Macrolides AMP* CST*	100% S 50% IR 100% R 100R 100% R
Bordetella bronchiseptica	1/1 XDR	Etiological agent of respiratory disease in pigs (anthropic rhinitis), dogs (Kennel cough) and rabbits (Snuffles), especially juvenile.	(62–64)	Cephalosporins Penicillins CPL in some horse cases SXT, S	CEQ, S - CTX, R AMC, IR- AMP, R CLP, S R – to 8/13 Aminoglycosides and Macrolides, SXT, TET & CST.
Mannheimia haemolytica	1/1	Primary bacterial species associated with BRD, responsible of significant economic losses to livestock industries worldwide. Commensal of URT of healthy cattle.	(65–67)	MDR highly prevalent	AMC, ERY, LS, TET & CST, R.

		Pneumonia and haemorrhagic septicaemia in sheep and buffalo. Isolated from human endocarditis, wound infection and septicaemia cases.			
Providencia rettgeri	1/1	Emerging nosocomial uropathogen, bacteraemia is uncommon but frequently found on urinary catheters causing UTIs. Isolated veterinary cases: cat hepatic disease and highly associated with alligator aquaculture facilities causing deathly encephalitis.	(68,69)	TET - R Penicillins* and cephalosporin. SXT – S CST* Decreased susceptibility to CIP S - newer cephalosporins	TET – S AMC*- R, AMP*-S STX – S CST – R CIP – R CEQ & CXT – S GEN* R
Rahnella aquatilis	1/1	Bloodstream infections, septic shock. Crucial for the spread of integron of AMR on aquatic environments. *R. aquatilis* harbours Class1 integron transferable to E. Coli strains that confers resistance to Aminoglycosides and SXT.	(70,71)	AMC - R Macrolides* - R Lincosamides*-R Aminoglyco. – S Penicillins – S Cephalosporin- S	AMC - S ERY – R LS – R GEN - S AMP – R CEQ – S CXT – IR CIP & ENR - R
Morganella morganii	1/1 XDR	Widely distributed in nature Commensal intestinal flora in human mammals and reptiles. Unusual opportunist pathogen mainly on post-OP wounds and UTI, neotal sepsis Some MDR strains cause sepsis with high mortality.	(72)	B-lactams* – R Cephalospor. – S GEN – S TET* - R Polimixins* - R	AMC & AMP – R CEQ – S, CTX – IR GEN - S TET – R CST – R SXT & CLP – S
Rhizobium radiobacter	1/1	Usually saprophytic, found in agricultural soils. Related with IV-catheter infections on cancer patients.	(73,74)	S to 3th Cephalos b-lactams, CIP and SXT. R to macrolides and amynoglyc.	AMC – IR AMP, CEQ, CTX- S STX – S ERY – R GEN – S, LS – R CST, CLP – R

** Intrinsic resistance (refer to Annex 1)*
*** Listing just those of relevance with this study.*
R (Resistant), IR (intermediate resistant), S (Susceptible) // BDR- Bovine Respiratory Disease // URT – Upper Respiratory Tract // LRT – Lower Respiratory Tract

All in all, our results and its further analysis show that all AMR present on these bacterial strains are considered by the WHO (75) as of Critical Importance and the vast majority to the highest priority of this group with similar considerations on the OIE standards for antimicrobial resistance (76). Furthermore, the all bacterial species found and its AMR characteristics belong to either the Critical or High priority WHO priority pathogen list (77).

CONCLUSION AND REMARKS

Taken together, the results of this study showed a very diverse population of bacterial species colonizing aquatic wildlife and with a great occurrence of those bacteria harbouring broad MDR profiles, XDR and PDR inclusive. Equally important was to notice that whereas *E. coli* stains were evenly distributed in reptiles and mammals, other species like *Aeromonas* spp and *Burkholderia cepacia* were isolated exclusively from the pond turtles, and the enterobacteria Morganella *morganii, Providencia* spp. and *Salmonella* spp. were recovered just form mammals. Regarding the AMR pools; MDR, XDR and PDR bacterial strains were equitable distributed between reptile and mammal hosts. For the most part, MDRB were mainly *Pseudomonas* and *Aeromonas* spp.; XDRs profiles mostly belonged to no-enterobacteria, namely *Burkholderia cepacia* and *Pseudomonas* spp, species which also corresponded to all identified PDR profiles. The most abundant resistant species of enterobacteria was *E. coli* in fact, >50% were XDR; also 100% of *Salmonella* recovered encounter XDR classification criteria.

As can be seen by the diversity of bacterial species and their extremely broad-spectrum pools of antibiotic resistance, a better understanding of the roles played by aquatic wildlife on the AMR dilemma is crucial. As reservoirs, they are silent keepers and melting pots of AMRs, additionally the width of their ranges and eminent contact with humans, pet animals or livestock makes then important vectors to be consider. However, is their beneficial protagonism as possible surveillance targets what is promising; for instance, our results together with the previous research consulted as part of this research, show that the bacterial species recovered have had clinical relevance on past years causing troubling MDR infections on animals and humans. Besides they mirror our MDR profiles, proving that monitoring aquatic wildlife, and specially those invasive species, might be the key source of information to enhance improved surveillance systems to capture the flow of AMR bacteria and genes on

the environment, plus it might serve as early warning of novel more challenging MDR profiles yet to be seen on the medicine field.

To conclude, future studies are needed which aim should be to correlate the environmental contamination sources with AMR genes clusters on fauna and ecosystems with an locations (GPS coordinates) tools that will allow mapping AMB spread and therefore find ways to stop or slow it down.

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ANNEX

ANNEX 1. Table of antibiotic categories tested on this study and the intrinsic resistant bacteria attributed to each one.

	Antimicrobial category	Antimicrobial Agent	Type	Mechanism Action	Application	Intrinsic resistant
Effects on bacterial cell wall and envelope.	Penicillin β-lactam antibiotic/ β-lactamase inhibitor	Amoxicillin/ Clavulanic acid	Bactericidal	Inhibit PBP cross-links/ bacterial β-lactamase inhibitors	Human Vet	*C.freundii, S.marcescens, M.morganii Providencia rettgeri*
	Aminopenicillins 3th generation	Ampicillin	Bactericidal	Irreversible inhibitor of the enzyme transpeptidase (bacteria wall)	Vet Human	*C.freundii Klebsiella spp. S.marcescens, M.morganii P.vulgaris Providencia rettgeri*
	4th Cephalosporin	Cefquinome	Bactericidal	Inhibit PBP cross-links	Vet ONLY	
	3th Cephalosporin	Ceftriaxone		Inhibit PBP cross-links	Vet Human	
	Polimixins	Colistin (Polimixin E)	Bactericidal	Hydrophobic/hydrophilic regions interact with the cytoplasmic membrane just like a detergent, solubilizing the membrane.	Vet Last-resort for multidrug-resistant Gram-negative infections, human.	*M.morganii P.vulgaris S.marcescens Burkholderia cepacia Providencia* spp
Inhibitors of nucleic acid.	Fluoroquinolones vet	Enrofloxacin	Bactericidal	Topoisomerase inhibitor	Vet	
	Fluoroquinolones 2th	Ciprofloxacin	Bactericidal	Topoisomerase inhibitor	Extra-label Vet use (ELU) Human	*Burkholderia cepacia*
Protein synthesis inhibitors	Aminoglycosides	Gentamicin	Bactericidal	Initiation inhibitors - 30S	Vet Human	*Providencia* spp.
	Lincosamides/Aminoglycosides	Lincomycin/ Spectinomycin	Bacteriostatic	Transpeptidation/translocation- 50S	Vet ONLY	
	Macrolides	Erythromycin	Bacteriostatic	Transpeptidation/translocation - 50S	Vet Human	*E.coli*

	Tetracycline antibiotics	Tetracycline	Bacteriostatic	tRNA binding- 50S	Vet Human	*Pseudomonas aeruginosa* *Proteus vulgaris* *M.morganii* *Providencia* spp.
	Amphenicols	Chloramphenicol	Bacteriostatic	Peptidyl transferase 50S prevents protein chain		*Burkholderia cepacia*
Stop Replication	Antifolates	Trimethoprim/ Sulfamethoxazole	Bactericidal	Purine metabolism, thereby inhibiting DNA and RNA synthesis	Vet Human	*Burkholderia cepacia*
* Includes only bacteria relevant for study. Table created based on the following information sources: (21,78–83)						

ANNEX 2. Proceedings diagram of data collection and analysis carried on by this study.